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Angiopoietin-2 interferes with anti-VEGFR2- induced vessel normalization and survival benefit in mice bearing gliomas

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Abstract

Purpose—In brain tumors, cerebral edema is a significant source of morbidity and mortality. Recent studies have shown that inhibition of VEGF signaling induces transient vascular normalization and reduces cerebral edema, resulting in a modest survival benefit in glioblastoma patients. During anti-VEGF treatment, circulating levels of angiopoietin (Ang)-2 remained high after an initial minor reduction. However, it is not known whether Ang-2 can modulate anti-VEGF treatment of GBM. Here, we used an orthotopic glioma model to test the hypothesis that Ang-2 is an additional target for improving the efficacy of current anti-VEGF therapies in glioma patients.

Experimental Design—To recapitulate high levels of Ang-2 in glioblastoma patients during anti-VEGF treatment, Ang-2 was ectopically expressed in U87 glioma cells. Animal survival and tumor growth were assessed to determine the effects of Ang-2 and anti-VEGFR2 treatment. We also monitored morphological and functional vascular changes using multiphoton laser scanning microscopy and immunohistochemistry.

Results—Ectopic expression of Ang-2 had no effect on vascular permeability, tumor growth or survival, although it resulted in higher vascular density, with dilated vessels and reduced mural cell coverage. On the other hand, when combined with anti-VEGFR2 treatment, Ang-2 destabilized vessels without affecting vessel regression and compromised the survival benefit of VEGFR2 inhibition by increasing vascular permeability. VEGFR2 inhibition normalized tumor vasculature while ectopic expression of Ang-2 diminished the beneficial effects of VEGFR2 blockade by inhibiting vessel normalization.

Conclusion—Cancer treatment regimens combining anti-VEGF and anti-Ang-2 agents may be an effective strategy to improve the efficacy of current anti-VEGF therapies.

Keywords

Angiopoietin-2; VEGFR2; brain tumor edema; vascular normalization

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Introduction

Anti-angiogenic treatments targeting VEGF signaling have led to both progression-free and overall survival benefits for patients with recurrent glioblastoma (GBM) (1,2). Preclinical and clinical studies suggest that alleviation of brain edema is primarily responsible for these promising results (2,3). Several potential biomarkers indicating benefit from or resistance to anti-VEGF therapy have also been proposed (4). For example, the Ang-1/Ang-2 ratio correlates with survival (5) and vascular normalization of GBM patients (6), whereas high Ang-2 levels correlate with resistance to anti-VEGF therapy (6). VEGF and Ang-2 are strongly induced by hypoxia in tumors. The induced Ang-2 destabilizes mature vessels, enabling VEGF to promote angiogenesis. A subsequent increase in Ang-1 levels and a decrease in Ang-2 levels stabilize the newly formed vessels. This concerted action of VEGF (7) and Ang/Tie-2 signaling also regulates vascular permeability. Induction of Ang-2 in tumor vessels not only increases permeability but also sensitizes endothelial cells to angiogenic or anti-angiogenic stimuli by destabilizing vessels (8). Therefore, depending on the microenvironment, increased Ang-2 expression can induce vessel formation or regression (Supplementary Table 1) and may affect the efficacy of anti-VEGF treatments.

Several studies have shown that a combination of VEGF targeting agents and vessel destabilizing agents is more effective than mono-therapies for causing vessel regression and inhibiting tumor growth (9,10). However, vessel-destabilizing agents, such as Ang-2 may create adverse effects in brain tumors where edema is a major concern. VEGF is a direct regulator of Ang-2 and the expression of Ang-2 has been linked to the inhibition of VEGF in colorectal cancer patients (11). In recurrent glioblastoma patients, blockade of VEGF signaling with the VEGF receptor tyrosine kinase inhibitor, cediranib, significantly reduced levels of plasma Ang-2 in some patients. However, this decrease was transient and modest (6) and its implications for anti-VEGF therapy remain unknown. In contrast to modest regulation of Ang-2 and the small benefit from anti-VEGF treatments in GBM patients, VEGFR2 blockade in our mouse glioma model was very effective at Ang-2 downregulation and conferred significant survival benefit. This sharp contrast in responsiveness to anti-VEGF treatments between GBM patients and our mouse glioma model, together with the correlation between Ang-2 levels and tumor progression in patients led us to hypothesize that Ang-2 regulation by anti-VEGF treatment contributes to the efficacy of anti-VEGF therapies.

To investigate the potential role of Ang-2 during anti-VEGFR2 treatment, we ectopically expressed Ang-2 in an orthotopic glioma model, mimicking regulation of Ang-2 seen in human glioma patients. We found that high levels of Ang-2 by ectopic expression destabilized vessels, interfered with vascular normalization by VEGFR2 inhibition and compromised the survival benefits of anti-VEGFR2 treatment.

Methods and Materials

Tumor model

Human Ang-2 was cloned into the retroviral vector, pBMNiGFP, and retroviral particles were packaged in HEK293ET cells. GFP or Ang-2 expressing U87 cells were established by retroviral transduction. To prepare source tumors for an orthotopic glioma model, 3 μ l of tumor cell suspension (5×10^5 cells) was injected into the cerebral cortex within cranial windows of male nude mice. The source tumors were cut into 0.5–1 mm diameter tumor pieces and implanted 1 mm deep in the cerebral cortex of recipient mice. All treatments were initiated when the tumor size reached about 2.5 mm in diameter. All animal procedures were performed following the guidelines of Public Health Service Policy on Humane Care of

Laboratory Animals and approved by the Institutional Animal Care and Use Committee of the Massachusetts General Hospital.

Tumor growth and survival

Tumors implanted in cranial windows were imaged using Zeiss Axioplan intravital fluorescence microscopy (Zeiss, Thornwood, NY) and tumor volume was calculated using the formula; $Volume = 0.5 \times Longer\ length \times Shorter\ length^2$. Control rat IgG or DC101 (anti-VEGFR2 antibody, ImClone Systems, New York, NY) was intraperitoneally injected every 3 days (40 mg/kg body weight). For Tie-2 inhibition, 10^9 pfu of AdExTek (a gift from Dr. Charles Lin, Vanderbilt University) (12) or AdLacZ virus were intravenously injected 24 hr before DC101 or IgG treatment. Survival experiments were terminated when mice became moribund or lost more than 20% of body weight or showed sign of paralysis.

Quantitative RT-PCR (qRT-PCR)

Total RNA extraction from tumor tissues was performed with TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RNA was further purified with RNeasy Mini kit (QIAGEN, Valencia, CA). cDNAs were synthesized using Taqman reverse transcription kit (Applied Biosystems, Carlsbad, CA) as described by the supplier. Quantitative PCR was performed using SYBR Green PCR master mix (Applied Biosystems, Carlsbad, CA) and specific primers. Primers sequences used are: mAngiopoietin-2 (forward) 5' GCATGACCTAATGGAGACCGTC 3', (reverse) 5' GATAGCAACCGAGCTCTTGGAG 3'; mVE-cadherin 5' (forward) GCTTCACTGTCAAGGTGCATGA 3', (reverse) 5' ATCTCTGGCACAGATGCGTTG 3'; mGAPDH (forward) 5'AAGAAGGTGGTGAAGCAGGCA 3', (reverse) 5' TGCTGTTGAAGTCGCAGGAGA 3'.

Permeability and Red Blood Cell (RBC) Velocity

The tumor area was localized by GFP signal constitutively expressed by U87 and U87-Ang2. Two adjacent areas were imaged by intravital multiphoton laser scanning microscopy (MPLSM) and 3D image stacks were acquired (3).

Vessel permeability to bovine serum albumin (BSA) was determined after i.v. injection of 0.1 ml (5 mg/ml) TAMRA-BSA or Alexa647-BSA (Invitrogen, Carlsbad, CA) as described previously (3). We alternated the use of TAMRA and Alexa647 conjugated BSA to minimize the residual background fluorescence. 10 minute after i.v. injection of BSA, Z stack images of 200 μ m with 2.5 μ m intervals were collected every 3–4 min up to 60 min. The increase in extravasated fluorescence dye intensity was normalized by blood vessel surface area. For RBC velocity analysis, we labeled red blood cells *ex-vivo* with a far-red lipophilic fluorescent dye (1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine perchlorate, DiD, Invitrogen, Carlsbad, CA), allowing observation deep inside the tissue via MPLSM. The labeled RBCs were mixed with the endogenous mouse blood via systemic injection at a ratio of 3–5 labeled RBCs per 100. Line scanning was performed using MPLSM to determine RBC velocity. All image analysis was completed using in-house algorithm (MATLAB, Mathworks, Natick, MA)(13). For more details of the data analysis, see Supplementary Methods.

Magnetic Resonance Imaging (MRI)

All MR images were acquired using a 9.4 Tesla MRI scanner (Bruker Biospin, Billerica, MA). Animals were anesthetized with a 50:50 mixture of O₂ and medical air plus 1.5% isoflurane and placed prone in a home-built cradle. A custom-built transmit-receive birdcage mouse-head coil was used to acquire the images. T2 relaxation maps were

generated from multi-echo spin-echo images and used to assess tumor edema. Acquisition parameters were: TE = 10 ms, 10 echoes, TR = 2500 ms, 11 image slices, 0.5 mm slice thickness, 150 μ m in-plane resolution, NA = 2. Voxelwise exponential fitting of the image signal intensity as a function of echo-time was performed using a MATLAB program written in-house to determine T2 relaxation time maps.

Immunohistochemistry and Western blot analysis

To label the perfused blood vessels, 100 μ g of biotinylated Lycopersicon Esculentum (Tomato) Lectin (Vector Laboratory, Burlingame, CA) was intravenously injected into mice, followed by perfusion fixation with 4% formaldehyde. 20 μ m thick frozen tissue sections were blocked in 5% non-fat milk in PBS with 0.1% Triton X-100 and stained with Alexa 647-conjugated streptavidin (1:200, S21374, Invitrogen, Carlsbad, CA). Pericytes were stained with Cy3-conjugated anti- α smooth muscle actin (α SMA) antibody (1:200, C6198, Sigma, St. Louis, MO) or anti-NG2 antibody (1:200, AB5320, Millipore, Billerica, MA). Ang-2 staining was done using polyclonal anti-Ang-2 antibody (1:100, AF623, R&D Systems, Minneapolis, MN) after heat retrieval at 95° C for 5min in target retrieval solution (S1699, DAKO, Carpinteria, CA). Images of four different fields per tumor section were collected with Olympus laser scanning microscope using 20X objective lens. Quantification of the stained area was performed using our in-house segmentation algorithm (MATLAB, Mathworks, Natick, MA). Image Analysis was performed as previously described (3). Images were processed using Adobe Photoshop CS3 software (Adobe Systems Inc., San Jose, CA).

Soluble Tie-2 expression was determined by Western blot analysis. Plasma samples (3 μ l each) from mice were separated on 8% denaturing polyacrylamide gel and transferred to PVDF membrane. The membrane was incubated with polyclonal anti-Tie-2 antibody (1:1000, AF313, R&D Systems, Minneapolis, MN), followed by horseradish peroxidase (HRP) conjugated donkey anti-rabbit IgG (1:5000, NA934V, GE Healthcare, Piscataway, NJ). The membrane was incubated in enhanced chemiluminescence (ECL) plus detection reagent (RPN2132, GE Healthcare, Piscataway, NJ) and exposed to Kodak ML film.

Data collection and Statistical analysis

Data are expressed as mean $\pm$ SEM. Student's t-test (two tailed with unequal variance) was performed for statistical analysis using Microsoft excel software. The Kaplan–Meier method was used for survival studies. We considered a *p* value of less than 0.05 to be statistically significant. For survival, permeability and MRI studies, all data from multiple experiments were combined, analyzed and presented. Other studies were repeated at least three times and representative data were presented.

Results

Anti-VEGFR2 treatment transiently reduces Ang-2 expression

We first determined the kinetics of Ang-2 expression during anti-VEGFR2 treatment. Ang-2 was predominantly expressed in tumor blood vessels of glioma xenografts (Fig. 1A) similar to patterns seen in autopsy samples from patients (14). Since Ang-2 was mainly expressed in vascular endothelial cells (ECs), we normalized the expression level of Ang-2 with respect to the EC specific marker, VE-Cadherin. As shown in Fig. 1B, DC101 treatment significantly, but transiently, reduced Ang-2 levels at day 2 followed by a gradual increase at later time points.

Ang-2 compromises the survival benefit from anti-VEGFR2 treatment

To investigate the potential role of Ang-2 during anti-VEGF therapy, we ectopically expressed Ang-2 in glioma cells (U87-Ang-2) to maintain high Ang-2 levels during DC101 treatment (Supplementary Fig. 1A). Testing for potential autocrine effects, we found that ectopic expression of Ang-2 did not affect growth of the cancer cells *in vitro* (Supplementary Fig. 1B). Also, there were no significant changes in the expression of angiogenesis genes as determined by an angiogenesis PCR array (SABiosciences, Frederick, MD) (Supplementary Table 3).

Next, we determined if Ang-2 expression or DC101 treatment affect tumor growth and animal survival. DC101 treatment significantly extended survival of glioma-bearing animals (Fig. 2A). Interestingly, tumor size at the end point was significantly larger in DC101 treated U87 tumors compared to control IgG treated tumors, suggesting that DC101 treatment contributes to longer animal survival by reducing edema-associated mass effects (Fig. 2B). In fact, DC101 had no effect on tumor growth (Fig. 2C). These findings are consistent with our previous study with cediranib (a VEGF receptor tyrosine kinase inhibitor) treatment in this model (3).

Next, we determined the impact of Ang-2 on U87 tumor growth and survival with and without DC101 treatment. Ectopic expression of Ang-2 did not induce a tumor growth delay or affect survival of tumor bearing mice (Fig. 2A, 2C). However, Ang-2 expression significantly reduced the survival benefit from DC101 (Fig. 2A). Ang-2 increased morbidity at a smaller tumor burden without affecting tumor growth (Fig. 2B and C).

Ang-2 impedes vascular normalization by anti-VEGFR2 treatment

Since anti-VEGF treatment can alleviate brain tumor edema and Ang-2 is also known to regulate vascular permeability, we measured permeability in tumor vessels to determine if edema contributes to the poor outcome in U87-Ang-2 tumors. Ang-2 expression alone did not change vascular permeability despite lower α -SMA positive mural cell coverage, indicating that VEGF signaling is the dominant regulator of vascular permeability in these tumors (Fig 3A and B). DC101 treatment significantly reduced permeability in U87 tumors at Day 2 (Fig. 3A) but did not reduce permeability in U87-Ang-2 tumors (Fig. 3B). Magnetic resonance imaging (MRI) confirmed that Ang-2 interfered with edema control by DC101 treatment (Fig. 3C and D). Importantly, vascular permeability positively correlated with the level of Ang-2 expression (Fig 1B and 3A). These data indicate that Ang-2 maintained vascular leakage during DC101 treatment, leading to additional tumor mass effects and morbidity in mice with smaller tumor burden.

To further characterize the impact of Ang-2 on tumor vessel function, we measured RBC velocity. Following DC101 treatment, the mean RBC velocity in U87 tumors increased at Day 2 suggesting that DC101 normalizes tumor vessels and improves their function (Fig. 4A). On the other hand, DC101 did not change mean RBC velocity in U87-Ang-2 tumors indicating that Ang-2 interferes with vascular normalization (Fig. 4B).

Next, we evaluated the morphological changes induced by Ang-2. Ectopic expression of Ang-2 resulted in dilated vessels (Fig. 5A), higher vessel density (Fig. 5B) and reduced mural cell coverage in U87-Ang-2 tumors (Fig. 5C, D and E). DC101 treatment effectively reduced vessel density in both U87 and U87-Ang-2 tumors, but U87-Ang-2 tumors consistently had higher vessel density than U87 tumors, even with DC101 treatment. We also found a gradual increase in mural cell coverage during DC101 treatment in U87 tumors but not in U87-Ang-2 tumors (Fig. 5D and E), consistent with the functional data showing that Ang-2 inhibits vessel normalization by VEGFR2 blockade. Interestingly, ectopic expression of Ang-2 did not increase vessel regression by DC101 treatment despite the

decrease in mural cell coverage (Fig. 5D and E), suggesting that Ang-2 did not sensitize blood vessels to VEGFR2 inhibition in this model. Collectively, our data suggest that the decrease in Ang-2 expression after DC101 treatment is critical for structural and functional vascular normalization.

Ang-2 does not antagonize Ang-1 in GBM

Since vessel function depends on the balance of Ang-1 and Ang-2 signaling through Tie-2, it is possible that Ang-2 interferes with DC101 by preventing Ang-1 from sending stabilizing signals through Tie-2. Another possibility is that Ang-2, by itself, signals for destabilization through Tie-2. To investigate this, we systemically expressed soluble Tie-2 (sTie-2) to block all signaling through Tie-2 (via both Ang-1 and Ang-2). sTie-2 expression reached a plateau within 24 hr and remained stable for at least 14 days after AdExTek administration (Fig. 6A). sTie-2 had no significant effect on tumor growth (Fig. 6B) or animal survival (Fig. 6C). Surprisingly, sTie-2 also had no significant effect on the survival benefit from DC101 treatment, suggesting that stabilizing Ang-1 signals do not contribute. Thus, the regulation of vascular permeability by Ang-2 may involve direct signaling rather than passive interference with Ang1/Tie-2 signaling. In fact, Ang-2 has been shown to increase permeability in cultured endothelial cells in the absence of Ang-1 or VEGF (15).

Discussion

In this work, we evaluated the potential of Ang-2 as a therapeutic target for improving anti-VEGF treatments. We found that high levels of Ang-2 limit the survival benefit from anti-VEGF therapy by interfering with edema control and vessel normalization. Brain edema is the most critical complication of brain tumors and the benefits of anti-angiogenic treatment appear to be primarily through relieving brain edema (3,6). Thus, our results suggest that Ang-2 inhibition in combination with an anti-VEGF agent may be a good strategy for controlling edema associated with brain tumors. Of note, Ang-2 levels have been shown to correlate with vascular leak syndrome in high dose interleukin-2 treatment of patients with metastatic renal cell carcinoma and melanoma (16). Along these lines, dexamethasone, a commonly used steroidal drug to control brain edema is known to suppress Ang-2 and VEGF expression (17,18).

Ang-2 expression is mainly associated with ECs in our tumor model as well as in GBM autopsy tissue (data not shown). Since we ectopically expressed Ang-2 in tumor cells in this study, it is possible that our tumor model does not completely reproduce human brain tumor biology. However, since Ang-2 is known to be readily diffusible and we did not detect any autocrine effect of Ang-2 on tumor cells, we suggest that tumor-expressed Ang-2 is an acceptable substitute for EC-derived Ang-2.

We found that Ang-2 increased tumor angiogenesis but did not affect the anti-angiogenic efficacy of a VEGFR2 inhibitor. Extensive vessel pruning by anti-VEGFR2 treatment did not slow tumor growth, suggesting that the remaining tumor vessels were sufficient to support tumor growth (3,19). However, it should be noted that the effects of ectopic expression of Ang-2 on tumor growth and angiogenesis depend on tumor cell type and tumor site (Supplementary Table. 1). This may be attributed to autocrine effects on tumor cells, endogenous levels of Ang-1 or other factors. Interestingly, Nasarre et al. reported that host-derived Ang-2 affects early stages of tumor development and vessel maturation but is dispensable at later stages of tumor growth (20). These results may explain why Ang-2 had no effect on tumor growth in our tumor model (Fig. 2), as we used fully established tumors in our study. On the other hand, similar to previous reports (21–23), we noticed a growth delay in U87Ang-2 tumors when we used cell suspension for tumor implantation (data not shown).

Using intravital imaging, we found that anti-VEGFR2 treatment functionally normalized tumor blood vessels – as demonstrated by reduced vascular permeability and increased RBC velocity - and Ang-2 compromised the vessel normalizing effect of anti-VEGFR2 treatment. These data confirm previous findings that Ang-2 destabilizes tumor vessels and promotes angiogenesis, while Ang-2 inhibition normalizes tumor vessels (24). However, Ang-2-mediated vessel destabilization did not increase the anti-angiogenic efficacy of DC101. In U87 tumors, DC101 treatment resulted in a gradual increase in mural cell coverage but changes in vessel function, i.e. permeability and mean RBC velocity, were temporary. In addition, tumor size did not reflect the benefit of treatment in brain tumors (3). Collectively, our results underscore the importance of functional measurements in evaluating the efficacy of any treatment in GBMs.

As in our tumor model, Ang-2 was transiently reduced in GBM patients after anti-angiogenic treatment (Fig 1A). One potential mechanism of transient nature of this decrease is the induction of Ang-2 by hypoxia caused by excessive vascular pruning. We tested this hypothesis by determining the expression of hypoxia-responsive genes. However, the hypoxia responsive genes, such as, CA9 and LOX, were not affected by DC101 treatment, suggesting that hypoxia is not the direct cause of Ang-2 regulation by anti-VEGFR2 treatment (Supplementary Fig 2A). Recently, G-CSF has been shown to be induced by vascular disrupting agents and to mediate the resistance to anti-angiogenic therapy (25,26). G-CSF also has the potential to induce Ang-2 expression (27). However, there was no significant change in G-CSF levels after DC101 treatment (Supplementary Fig 2B). DC101 treatment increased mural cell coverage of tumor vessels, which could render the endothelium resistant to anti-angiogenic treatment. It is, therefore, possible that vessels inherently resistant to DC101 treatment and mature vessels that survived during DC101 treatment continue to produce Ang-2 in a VEGFR2 independent manner, which could be a potential bounce back mechanism of Ang-2 expression during DC101 treatment.

The important implication of our study is that in tumors where Ang-2 levels remain high during anti-VEGF treatment, the efficacy of anti-VEGF therapy might be limited. Therefore, the modest reduction of Ang-2 seen in glioblastoma patients treated with cediranib might partly account for the limited efficacy of anti-VEGF therapy. Our findings support the recent studies - performed in ectopic tumor xenograft models - suggesting that Ang-2 is a viable therapeutic target (Supplementary Table. 2) and that simultaneous inhibition of VEGF and Ang-2 pathways is more effective than inhibition of either pathway (28).

Translational relevance

Bevacizumab, an anti-vascular endothelial growth factor (VEGF) antibody, has recently been approved for the treatment of recurrent glioblastoma(rGBM). However, the survival benefits from anti-VEGF treatment are modest and additional targets for anti-angiogenic treatment are urgently needed for rGBM. In GBM patients, Angiopoietin (Ang)-2 levels correlate with survival and are associated with tumor vascular normalization and resistance to anti-VEGF therapy. However, the effects of Ang-2 in the context of anti-VEGF treatment have not been explored in GBM. Here, we investigated the role of Ang-2 during anti-VEGF treatment and found that high levels of Ang-2 interfered with vascular normalization induced by VEGFR2 inhibition; this compromised the survival benefits of anti-VEGFR2 treatment. Our findings imply that the efficacy of anti-VEGF therapy can be compromised if Ang-2 levels are high and thus, Ang-2 inhibition is a worthwhile addition for improving the outcome of current anti-VEGF therapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Expression of Ang-2 during VEGFR2 inhibition

A. Untreated tumor tissue sections were stained for ECs (Red) and Ang-2 (Green). Ang-2 was dominantly expressed by ECs. Scale bar, 50 μ m. **B.** Ang-2 and VE-Cadherin transcript levels were semi-quantitatively determined by real-time PCR. Ang-2 expression was transiently reduced by DC101 treatment. N=5.

Figure 2. Effect of Ang-2 and VEGFR2 inhibition on tumor growth and survival

A. Tumor bearing mice were treated with IgG (dotted line) or DC101 (solid line) and followed for their survival. DC101 treatment significantly extended survival of mice, and Ang-2 compromised the survival benefit of DC101. **B.** DC101 treated mice (filled circle) survived with significantly larger tumors compared to untreated mice (open circle), but not Ang-2 tumor bearing mice. **C.** Neither Ang-2 nor DC101 treatment had an effect on tumor growth. Purple and brown lines indicate IgG and DC101 treatments in control tumors. Light blue and blue lines indicate IgG and DC101 treatments in U87-Ang-2 tumors. Each line represents an individual mouse.

Figure 3. Permeability changes in tumor vasculature by Ang-2 and VEGFR2 inhibition

A. DC101 treatment decreased vascular permeability temporarily in control tumors. N= 8 (Ig G) and 9 (DC101). **B.** Ang-2 interfered with the effect of DC101 on vascular permeability. DC101 treatment did not reduce the vascular permeability in U87-Ang-2 tumors. N=7 (IgG) and 8 (DC101). **C.** MR imaging was performed before (Day 0) and 2 day (Day 2) after treatment with IgG or DC101. Representative T2-weighted images were shown. Arrows indicate the tumors grown in cranial windows. Scale bar, 2mm. **D.** DC101 treatment was more effective in reducing tumor edema in U87 tumors than in U87Ang-2 tumors. N= 7.

Figure 4. Effect of Ang-2 and VEGFR2 blockage on RBC velocity

A. Average RBC velocity was temporally increased by DC101 treatment in control tumors. The regulation kinetics of RBC velocity correlated with kinetics of vascular permeability and Ang-2 expression. **B.** DC101 treatment had no effect on RBC velocity in U87-Ang-2 tumors. Left panels show average velocity data and right panels show the data from individual animals. N=5.

Figure 5. Effect of Ang-2 and VEGFR2 blockage on tumor blood vessels

A. Tumor vasculature was imaged using MPLSM after the injection of rhodamine conjugated dextran and DiD labeled RBC. Green color shows the perfused vessels with rhodamine conjugated vessel and Red color shows labeled RBCs. Vessels were dilated in U87-Ang-2 tumors compared to control tumor vasculature. DC101 treatment had no significant effect on vessel diameter. Mean vessel diameters were determined from Z stack images collected using MPLSM. Scale bar, 200 μm . B. Ang-2 increased total vessel length. DC101 treatment was equally effective in vessel trimming in both tumors. C. Immunohistochemical analysis was performed and images were collected using confocal laser scanning microscopy. The representative images of blood vessels and pericyte coverage after VEGFR-2 inhibition are shown. Blood vessels and pericytes from the frozen sections were stained for lectin (Green) and αSMA (Red), respectively. D. Ang-2 decreased αSMA positive fraction in vessels. DC101 treatment increased αSMA positive cell coverage in control tumors but not in U87Ang-2 tumors. E. Ang-2 had no effect on NG-2 positive fraction while interfering with the increase in NG-2 positive vessel fraction by DC101 treatment. DC101 treatment increased αSMA positive cell coverage only in control tumors. N=8 (Day 0 and 2) and 4 (Day 8).

Figure 6. Effects of Tie-2 inhibition on tumor growth and mouse survival with VEGFR2 inhibition

A. Single intravenous administration of ExTek adenovirus maintained the sTie-2 expression for more than 2 weeks. #; animal number. **B.** sTie-2 had no effect on tumor growth in IgG (left panel) or DC101 (right panel) treated mice. N=6. **C.** sTie-2 had no significant effect on the survival benefit of VEGFR2 inhibition.